AMENDMENTS

AMENDMENTS TO THE SPECIFICATION:

Amend the specification, excluding claims, as follows:

Molecular-Biological Marker for Analytical Electron Microscopy

This application receives priority from application PCT/DE00/00116, filed January 07, 2001, which claims priority to German Patent Application No. 199 00 511.7, filed January 07, 1999, the entireties of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

The invention relates to plasmids derived from pBluescript KS(+), comprising more than 1 SK primer sequence element, preferably 2, 7, 14, 21 and 27 repetitive SK primer sequence elements, and their use as molecular-biological markers in analytical electron microscopy.

Electron spectroscopic imaging (ESI) is a method of analytical electron microscopy (EM), which pictures the distribution of **[[a]]** certain chemical **elements** within **an [[the]]** analyzed preparation. In order to elucidate the structural organizations of biological systems, it must be possible to optically differentiate the individual macromolecular components. At present, **[[the]]** charging with gold particles or other particles, which are visible in **[[the]]** refraction contrast, is used to label macromolecules for electron microscopy.

Multiple labeling experiments have been carried out in electron microscopy**, where ~~by using grains~~ particles** of various sizes **are used, which enable differentiation of ~~to be able to differentiate the~~** different target structures in a single preparation. For example, one **type of** molecule **~~type would be~~ is** linked to gold **~~grains~~ particles** having

a size of 5 nm while **another type is linked to** ~~**the other would be attached to those having a size of**~~ 10-20 nm **sized particles** in a double labeling **experiment,** to ensure that in a subsequent evaluation **[[the]]** different molecules can **be** clearly **[[be]]** localized and distinguished from one another. Large gold ~~**grains**~~ **particles** (larger than 10 nm) are **disadvantageous,** because **of lower** ~~**they have reduced**~~ penetration capacity into the tissue and reduced coupling efficiency to **a** **[[the]]** target molecule (Giberson, T.R., and Demaree, R.S.: The influence of immunogold particle size on labeling density. Microscopy Research and Technique, 27, 355-357, 1994). In addition, such a large **particle is** ~~**structure can**~~ no longer **[[be]]** assigned ~~**clearly to the site of**~~ **to a** binding **site of a** ~~**to the**~~ target structure, **[[i.e.]]** **because,** resolution capability **would be [[is]]** lost. If a triple labeling experiment **[[was]]** **is** aimed at, **the above** **[[these]]** drawbacks would become particularly striking. Only ~~**what is called**~~ ferritin molecules, i.e. large protein units, which contain hundreds of iron atoms in their centers and can be linked to target structures, **which** are **[[an]]** alternative to the gold **particles**. However, their electron density and ~~**their**~~ detectability under the transmission electron microscope is very poor**. Therefore,** ~~**so that**~~ their use has **been** **[[only]]** proved feasible **only** in rare cases.

On the other hand, florescence methods enable triple and quadruple labeling without ~~**causing**~~ **any** major problem**[[s]]** in optical microscopy. Electron microscopy with the existing labeling techniques could not compete with optical microscopy. Therefore, scientists have been using optical microscopes with comparatively poor resolution capability. The development of **an** alternative labeling technique**[[s]]** for gold labeling would render electron microscopy more **attractive,** because its advantageous labeling**[[,]]** provides a resolution capability over 100 times as good as that of **the** optical microscopy. The gold labeling method for conventional transmission electron microscopy is based on the electron density of ~~**the**~~ heavy metal gold and there is a demand for **an** alternative labeling method**[[s]]** for ESI. This technique**, which** utilizes interactions between beam electrons and the atoms in the preparation**, differs from** ~~**differing from those of**~~ conventional transmission electron microscopy. In principle, all of the elements can be detected specifically**, which.** ~~**This**~~ raises the number of

elements in consideration for labeling methods. However, to establish alternative labeling methods, it is decisive to check detection limits for the elements in consideration. This means~~, in concrete terms, that~~ information is required on the number of detectable element atoms per nm^2 area~~ in the preparation~~. Therefore, the detection limits of the ESI technique are relevant. Only a few study-reports and vague indications on this parameter are available. Although the ESI technique is often used, no data on detection limits have been published to date.

There is a demand for alternative labeling methods for electron microscopy. It should be possible to readily test and assess the detectability of ~~such~~ a marker complex.

Therefore, an object of the present invention is to provide a method of obtaining data, to evaluate the prospects of the intended experiment with the element in question and/or the marker structure in question, before time-consuming cytobiological and molecular-biological experiments are carried out. Furthermore, the parameter for the detectable number of elementary atoms per unit area shall become measurable to obtain necessary information to establish EM labeling methods.

This object is achieved by the subject matters defined in the ~~attached~~ claims **section**.

~~The reason why the above-mentioned preliminary tests are necessary is that so far~~ **Until now, there has been** no accurate limiting values of detectability ~~have been~~ known for the ESI detection of the various chemical elements. This is *inter alia* due to the fact that preparing a suitable test sample is not a trivial matter. Such a sample must have special properties. There must be regions in which the target element is available in a clearly defined amount. It must be possible to clearly detect these regions. The target element may not occur in the remaining regions. This problem has been reported by investigators who tried to record the resolution and detectability by means of grainy precipitates, using uranium**, for** ~~as an~~ example (see, Golla and Kohl, Micron, 28:(5), 397-406, 1997).

BRIEF SUMMARY OF THE INVENTION

In one aspect, the invention provides plasmids derived from pBluescript KS(+), comprising more than 1 SK primer sequence element, preferably 2, 7, 14, 21 and 27 repetitive SK primer sequence elements.

In another aspect, the invention provides methods of ~~detecting a target structure by~~ analytical electron microscopy, comprising: providing the plasmid as described herein; adding **a detectable element to the SK primer, thereby forming** a marker **complex** ~~to the plasmid to form a plasmid-marker complex; binding the plasmid-marker complex to the target structure~~; and imaging the ~~bound~~ **marker** complex by electron microscopy.

In yet another aspect, the invention provides a test kit for use in electron microscopy comprising: host *E. coli* JM110 bacterial cells suitable for replicating the plasmid as described herein; and a single-stranded plasmid comprising 2, 7, 14, 21, and 27 repetitive SK primer sequence elements.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows construction of pBl KS(+) containing 2 repetitive SK primers. The multiple cloning site (MCS) and the SK primer sequences are shown in boxes. Restriction sites are marked with broken lines. The figure also shows sequence segments that are important for cloning.

Figure 1A. Diagram of pBl KS(+) (SEQ ID NO:2): pBl KS(+) is digested with the restriction enzymes Kpn I and BamH I for subsequent cloning. The restriction sites are marked with broken lines. The MCS fragment is flanked by the restriction sites.

Figure 1B (SEQ ID NOs:2 and 3) shows pBI KS(+) digested with BamH I and Kpn I and a SK-PH I fragment resulting in pBI KS(+) 2 repetitive SK when ligated with pBI KS(+). Part of MCS is excised by digestion with Kpn I and BamH I (also see Figure 1A), and fragment SK-PH I is inserted. Previously present Bam H I restriction site is masked by SK-PH I via a base pair modification (bold letters), while a new BamH I restriction site is introduced at the same time. Due to the different restriction sites (Kpn I/BamH I), the fragment can be cloned only in one orientation. The restriction site Pvu I served as a control restriction site for the successful incorporation of the insert SK-PH I.

Figure 1C shows a diagram of pBI KS(+) containing 2 repetitive SK primers (SEQ ID NO:4). pBI KS(+) containing 2 repetitive SK primers is constructed by ligation of SK-PH I with the BamH I/Kpn I digested pBI KS(+) (also see Figure 1B). The modified BamH I restriction site, marked by an asterisk, could no longer be excised by BamH I. SK primer and non-hybridizing sequences are indicated by an arrow. This leads to the schematic plan for pBI KS(+) containing 2 repetitive SK primers as shown in Figure 1D.

Figure 1D shows a simplified diagram of pBI KS(+) containing 2 repetitive SK primers. Solid line represents MCS and broken lines indicate the remainder of vector pBI KS(+). Arrows show the 5' → 3' direction of the cloned SK primers plus a 4 bp sequence, which is a non-hybridizing fragment (also see Figure 1C).

Figure 2 shows a simplified diagram of vector pBI KS(+) containing 7 repetitive Sk primers (SEQ ID NOs:10 and 11). The pBI vector is marked by a broken line; seven SK primer sequences are located at Kpn I/Sac I-oriented MCS. The SK-PH II fragment (dotted arrow on the top and the sequence of "SK-PH II" is emphasized by lines at the bottom) inserted the seventh SK primer and the additional Eag I restriction site in the vector. Restriction sites are marked on the sequence.

Figures 3A and 3B show diagram of cloning a pBI containing 2 repetitive block plasmid.

Figure 3A shows insertion of a 7 repetitive SK block in the Not I- digested pBI 1x block DNA. Descriptions of the individual components are as shown in Figures 1 or 2. The clone pBI 1x block is linearized with the Not I enzyme and ligated with the PCR fragment subsequently cut by Eag I beforehand.

Figure 3B (SEQ ID NO:12) shows the transitions between individual blocks. The construction of the components is similar that of Figures 1A-1D. By ligation of the 7x SK block (gray arrow) in the proper orientation, the Not I restriction site, which digested the pBI 1x block beforehand is masked by the 5' end of the newly added 7x SK block (bold letters) and could no longer be excised by Not I. The 3' end of the fragment completes the Not I restriction site towards the vector. As a result, it is possible in the subsequent cloning run to linearize the pBI 2x block with Not I without losing the 14 SK primer. In contrast to the BamH I cleavage site between the individual SK primers in the block (BamH I*), the BamH I restriction site at the 5' end of a 7x block is maintained (BamH I) and can subsequently be used as an orientation control.

Figure 4 shows the resulting sequence of the plasmid construct containing 27 repetitive SK primer elements (SEQ ID NO:1). Solid lines on the top indicate the SK primer sequence regions in the repetitive region, which are sequenced from both ends. Sequences ATCT or GCCG having a length of 4 base pairs are located between these SK primer sequence regions for reasons of cloning technique.

~~**Figure 5.**~~ Figure 5 shows, as an outline, an overall diagram of a plasmid (Solid circular line) that contains 16 repetitive SK primers (16 small solid circles), to which ESI markers bind; and, in greater detail, a diagram of two 20-nucleotide long repetitive SK primer fragments (the lower strand) that are separated by a spacer of a 4-oligonucleotide long fragment (see a gap on the lower strand). ESI markers (the solid balls) are covalently

bonded with the single-stranded oligonucleotides (upper strands). The oligonucleotides (upper strands) are bound by complementary base pairing (as a result of hybridization) of the repetitive SK primer fragments (the lower strand) on the plasmid.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides ~~**According to the invention**~~ a series of new plasmids having more than 1, preferably 2, 7, 14, 21 and 27, SK primer sequence elements, was produced in direct head/tail-oriented repetition on the basis of pBluescript KS(+). The ~~**annularly closed**~~ plasmid**s present** ~~**is available as**~~ a target ~~**structure**~~ **structure,** which contains repetitively a short DNA sequence (**the** SK primer sequence element). The SK primer sequence element comprises the following sequence (SEQ ID NO:5):

5'-GATCCACTAGTTCTAGAGCG-3'.

The SK primer **(Stratagene)** sequence represents a segment of 20 nucleotides ~~**which is referred to as such by the company of Stratagene**~~ in ~~**the**~~ vectors pBluescript KS(+/-) and Bluescript SK (+/-) **(Stratagene), which is located** ~~**and lies**~~ within the "multiple cloning site" (MCS) **of the vector**. pBluescript is a plasmid vector sold by Stratagene. It is ~~**an annular**~~ **a circular** DNA molecule ~~**which**~~ **that** contains ~~**the**~~ genetic **materials required** ~~**information necessary**~~ for replication in *E. coli*. The multiple cloning site (MCS) is decisive for cloning foreign DNA segments into **[[this]] the** vector. According to **an embodiment of** the invention the above-mentioned SK primer sequence repetitions were incorporated into **[[this]] the** MCS region so that the resulting plasmids of pBluescript only differ ~~**as regards**~~ **in** the MCS, i.e. **the plasmids** are derived from **the** pBluescript.

A homologous sequence may **bind the** ~~**be bound to this**~~ repetitive sequence by ~~**means of**~~ hybridization. In **the event, that a** ~~**case this**~~ hybridizing sequence carries a marker complex, the marker will reach the target structure by hybridization.

The hybridizing sequence, ~~hereinafter referred to as~~ **which refers to** SK oligonucleotide or SKO, can be modified chemically at its ends to permit a covalent ~~bond~~ **binding** of different markers. As a result, it is possible to study any labeling strategies. A molecule can be linked to the SKO containing an element in the highest possible concentration**, which should be** ~~which shall be~~ tested for usability as a marker for ESI. Boron **markers,** as described in German patent application 198 03 206.4, for example, are **suitable** ~~suited~~. Further promising markers are **silicon,** ~~as well as~~ iron and manganese. The marker compound is built up in a controlled synthesis such that the number of target elementary atoms is known and the target element is present in the greatest possible amount in the center of the marker compound. It can also be linked to the SKO as a unit. In order to **do that,** ~~meet these demands, e.g.~~ the boron marker structure is synthesized by treating it like a nucleoside unit in the oligonucleotide synthesis**, for example**. ~~Thus~~**Therefore**, the preferred linking system provides the production of a boron compound containing the necessary protective groups and linkage groups for the oligonucleotide synthesis according to the phosphoramidite method**. During the process**, ~~in the course of which~~ an oligonucleotide is built up, building block by building block, from the 3' end to the 5' end. ~~In this connection, there is the~~ **There is a** possibility that for this purpose the boron complex is attached as such or in the form of a 5'-boron nucleotide(C)-3' building block to the 5' end of SKO ~~in the last~~ **at the final** step (see ~~in this connection also~~ German patent application 19803206.4**, for example**). It is advantageous for the marker-containing building block to contain a spacer which will cause the marker complex to stand off from the SKO so as not to impede the hybridization of the labeled SKO with the complementary plasmid regions. Aliphatic hydrocarbon chains which have lengths between C_2 and C_{10} and may possibly contain oxygen groups in the form of ether bridges (preferably a maximum of 5 bridges) are ~~in consideration~~ **considered** as spacers (see ~~in this connection also~~ German patent application 198 03 206.4**, for example**). A similar procedure can be used for any other marker structures~~, each~~ containing ~~another~~ **a** target element.

The labeled oligonucleotides are hybridized to the DNA and are present selectively in the preparation at the sites where the ~~annular~~ **circular** DNA molecules are located.

Depending on the **number of repetitions** ~~**repetition degree**~~ of the SK elements on the employed plasmid molecules, variable but defined amounts of target elementary atoms ~~**will thus be**~~ **is** attached to the DNA in a very close arrangement. Therefore, densest packaging can be assumed because it was found that the distances of the marker structures on the DNA are 8 nm. This follows from a calculation of the extension of double-stranded DNA regions over the SK repetition units present in the plasmids. Since the marker structure ~~**will have**~~ **contains** a maximum diameter of 5 nm there is in fact some space available between the markers. However, this space should be maintained because the hydrate envelope of the marker compounds must be taken into account.

The plasmid according to the invention is prepared on the carrier matrix of the sample holder for ESI in ~~**spread**~~ **linear** form. The above plasmids enable the preparation of single-stranded ~~**annular**~~ **circular** plasmid DNA molecules after infecting plasmid-containing *E. coli* cells, preferably *E. coli* JM 110, by ~~**means of what is called**~~ a helper virus. The (+) sign in the name of the original plasmid pBluescript KS (+) indicates that only the plus strand of the plasmid molecule is isolated. A single-stranded DNA sample is **[[now]]** **therefore** available ~~**against which**~~ **to** complementary DNA regions**, which can be readily** ~~**can readily be**~~ hybridized without ~~**the otherwise necessary**~~ fusing **[[of]]** the DNA duplex. In order to hybridize SK oligonucleotides (SKO) complementary with the plus strand of the plasmids, they must, of course, represent the sequence of the minus strand, i.e. 5'-CGCTCTAGAACTAGTGGATC-3' **(SEQ ID NO: 3)**. Such an oligonucleotide can be produced by ~~**means of**~~ **an** automatic oligonucleotide **synthesizer** ~~**synthesis**~~. These molecules are mixed in an aqueous solution with one of the above-mentioned single-stranded plasmid molecules. Double-stranded regions form at the sites where the SK oligonucleotides (SKO) have found the complementary partner on the singled-stranded DNA, i.e. SK oligonucleotide/plasmid hybrids (~~**hereinafter referred to as**~~ SKOPH). In order not to impede the binding of the single SKOs to the DNA, a gap of 4 nucleotides is preferably provided as a spacer between the SK oligonucleotide binding sites.

These SKOPHs are preferably separated by the chromatography of unbound SKOs. This may be done by column chromatography, e.g. Amersham Pharmacia Biotech (Freiburg, Germany) offer column matrixes (e.g. sephadex or sepharose). The purified SKOPHs are then subjected to spreading. In this connection otherwise coiled DNA molecules are pretreated such that they are stretched in solution and in this state are applied onto electron-microscopic small carrier nets coated with a thin sheeting, made visible by treatment with heavy metals and analyzed under a transmission electron microscope (TEM). If ESI analysis shall be carried out, heavy metal treatment should be dropped, since every element occurring in high amounts and/or high concentrations in the preparation interferes with, or makes impossible, the specific detection of the target element. The DNA rings are then distributed uniformly over the surface of the TEM preparation and are separate from one another. When above-mentioned basic preconditions are met: the ~~annular~~ **circular** DNA is clearly evident, the SKOs are available in a more or less large number and are bound to the DNA, and there is (almost) nothing in between the DNA regions.

In case **if** it is not clear whether the SKOs were bound to the repetitive region **or not**, there are two possibilities for control: a) SKOs labeled at the 5' position by digoxigenin or biotin are used against which an anti-digoxigenin or an anti-biotin antibody is ~~employed~~ **employed,** which itself is labeled with gold and can be detected by conventional TEM. The size of the gold ~~grains~~ **particles** may, however, not exceed a diameter of about 6 nm (otherwise the gold ~~grains~~ **particles** could interfere with one another); b) the repetitive target plasmid can be linearized, possibly in combination with a), by restriction endonuclease digest along with the repetitive SK region, so that following spreading the binding sites of the SKOs are readily identifiable in that they must be located at the end of a thread-like DNA molecule. Since restriction endonucleases only excise double-stranded DNA, its restriction site must first be made double-stranded by hybridization of an oligonucleotide complementary around the restriction site.

The repetitive sequences are arranged closely one behind the other and extend over about a third of the plasmid. These repetitive sequences render the test much more significant. The advantage of the above described plasmids consists in that 1 to 27 of the marker units can be accumulated so as to modulate the number of marker elementary atoms. When it is possible to show the labeled SKOPHs in differing spreading states from fully extended to coiled in the spreading preparation, the target elementary atoms, bound particularly to coiled DNA molecules, can i) be concentrated within a very confined space, ii) become localizable due to the uniformly fibrillary ring shape of the bound-DNA, iii) be analyzed in defined but variable number, and iv) be in an otherwise element-free environment.

DNA segments outside the repetition regions to which no marker can bind serve as a negative control for the ESI elemental detection. Such a negative control is necessary because the specificity of a calculated target element distribution could be doubted if there **[[was]]** **were** no comparative region without target element and correspondingly without calculated element signal. Since the test represents a molecular-biological system, the marker is assessed in its physico-chemical environment. This also means that the test is very close to a medical/biological application, in particular the *in situ* hybridization.

It is an objective of this test method to obtain reliable data on the minimum number of target element atoms per unit area necessary for ESI detection. At the same time, data are obtained on the individual detectability of the marker structure and because of the repetition, it is also possible to obtain average weak element-specific signals, in particular, in DNA molecules available in the electron-microscopic preparation in a fully stretched manner. Therefore, it can be determined prior to a technically complicated use of a marker structure in medicine or biology whether optionally the number or/and the concentration of the target elementary atoms must still be increased in the marker structure. All of the plasmid states from stretched to considerably coiled are found in spreading preparations, in particular when the spreading process did not proceed in

optimum fashion. This is of advantage in connection with the explained determination of elemental detection limit.

Many variations of spreading methods are found in the literature (~~regarding a summary see:~~ see for example, Electron Microscopy in Molecular Biology; a practical approach, Sommerville, J. and Scheer, U. (eds.), IRL Press, 1987).

The threshold values for the element-specific detection can be determined by the standard methods of elemental detection using ESI. For this, there is presently no other method available. Therefore, it can be considered that this method is also of interest for the scientists who do not have a bio-medical application in mind, ~~in mind a biologically/medical use~~ but are interested in the detection limits of any chemical elements other than those mentioned above. The precondition is that the target element is already present in the marker structure linked to the oligonucleotide in the highest possible concentration and in the greatest possible amount.

The use for ESI ~~has been specified~~ is described above. In addition, applications of parts of the test system are also ~~possible~~ possible, which go beyond the use in electron microscopy. Two ~~further~~ additional sample applications are ~~mentioned here~~ briefly described ~~and specified~~ below: 1) the SK primer repetition cassette can generally be utilized for [[the]] an efficient and localized DNA labeling by hybridizing with labeled oligonucleotides; and 2) for studying the mechanisms of deleting direct repetitions in DNA, as described below ~~the below described plasmids form the model substrate~~.

In addition to the application in the field of electron microscopy, said repetition regions enable, as stated above, the possibility of also rendering DNA quite generally detectable after the hybridization by means of the SKO-linked markers which are still below the detection limit as single molecules but can be identified in repetitive arrangement. For this purpose, the repetition regions can also be recloned into the desired DNA molecules via SAC I/Kpn I-compatible ends. For example, the route of DNA can then be

tracked by such a method after introduction into a cell (transfection). Here, the uses of both optical and electron microscopes are in consideration.

The chemical modifiability of the hybridizing sequence permits different uses of the test for differently configured marker units. Since the test represents a molecular-biological system, the marker is assessed in its physico-chemical environment. The individual detectability of the marker is analyzed. The intensity of weak signals can be determined precisely by averaging.

The following statements made on the production of the plasmids containing different repetitions show that in principle the repetition steps (pBluescript KS (+)) 2x, 3x, 4x, 5x, 6x, 7x, 14x, 21x and 27x SK are available for the experiments for the actual elemental detection for ESI. Since the analysis of differences as to the detectability of the target element using ESI will be especially convincing when the number of analyzed elementary atoms varies considerably (~~see in this connection the below statements~~ **see description below**), the **number of repetitions** ~~repetition degrees~~ 2x, 7x, 21x and 27x SK are of special interest.

As mentioned above, the present invention is based on the fact that a test preparation contains areas in which the target element is available in a clearly defined amount and can be detected unambiguously. The target element may not occur in the remaining areas.

The plasmid construction is stabilized by introduction into a dam^-/dcm^- strain (preferably *E. coli* JM 110). JM110 is dam^-/dcm^- and contains no other striking genotypic markers, which would clearly distinguish this strain from the other ones used, so that they can be employed as well. The repetitive plasmids according to the invention are introduced into the dam^-/dcm^- strain according to standard methods (*cf.* Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular cloning; A laboratory manual; Second edition, Cold Spring Harbor Laboratory Press (1989)). Surprisingly, a deletion of the directly repetitive elements is avoided during bacterial replication. It is known that the direct repeats or

inverted repeats are lost during the replication in *E. coli*. Dam/dcm strains are documented in the literature (*cf.* Marinus *et al.*, J. Bacteriol. 114 (3), 1143-1150 (1973)); however, stabilization of directly repetitive sequences~~, resulting therefrom,~~ has never been described.

The **number of repetitions** ~~repetition degree~~ could even be increased to 27x in *E. coli* JM110. Furthermore, the combination of *E. coli* JM110/pBI KS (+) 27x SK is ~~for the first time~~ a **new** system in which a direct repetition sequence otherwise unstable in *E. coli* can be replicated. Bacterial geneticists get the possibility of analyzing the underlying mechanisms of this type of deletions in bacteria and characterize the involved components. The question of stabilizing repetitions of such a type in *E. coli* is of interest e.g. for cloning specialists who try to obtain human DNA segments in their original state even if they had been replicated in *E. coli* (see e.g. human genome project). The background is that human DNA segments also contain short directly repetitive segments which like the above-described SK primer sequence repetition can show relatively poor stabilizing characteristics.

The plasmids according to the invention can be combined into test kits for use in electron microscopy. A test kit contains e.g. the following materials: 1) competent *E. coli* JM110 bacterial cells for replicating the repetitive plasmids; 2) the single-stranded plasmids **containing** 1x or 2x, 7x, 14x, 21x and 27x SK for the differential analysis of marker structures for the electron microscope; 3) electron-microscopic small carrier nets which are already coated for spreading; 4) SK oligonucleotides labeled at their 5' end by biotinylation or digoxigenation and serving for optimizing hybridization and spreading by proving by means of a gold-linked anti-biotin or anti-digoxigenin antibody that the repetitive arrangement is actually given on the plasmid; **and** 5) instructions describing the individual processing steps. If there is an interest in applications other than the ESI-dependent ones, the test kit can be modified ~~for those interested~~.

The plasmids having 2, 7, 14, 21 and 27 SK primer sequence elements were deposited as *E. coli* cultures with DSMZ **(German-type collection of microorganisms and cell**

cultures) ~~(*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*) [German-type collection of microorganisms and cell cultures]~~, Mascheroder Weg 1, Braunschweig, Germany, under accession numbers DSM 12600, DSM 12601, DSM 12602, DSM 12603, and DSM 12604 on December 22, 1998:

pBI KS(+)2xSK	DSM 12600
pBI KS(+)7xSK	DSM 12601
pBI KS(+)14xSK	DSM 12602
pBI KS(+)21xSK	DSM 12603
pBI KS(+)27xSK	DSM 12604

~~The following figures explain the invention in more detail.~~

~~Figure 1: Outline of the production of pBI KS(+) 2x SK.~~
~~The kind of presentation given here is continued in the following illustrations of this kind. The multiple cloning site (MCS) is shown as a dark-gray block, and the SK primer sequence included therein is light gray. The restriction sites are marked by a finely broken line. The detailed sequence is indicated by the segments important for cloning.~~

~~a) Diagram of pBI KS(+). PB1 KS(+) was digested with the restriction enzymes Kpn I and BamH I for subsequent cloning. The restriction sites are marked by a finely broken line. The MCS fragment therebetween falls out.~~

~~b) Diagram of pBI KS(+) digested with BamH I and Kpn I and the SK-PH I fragment which should result in pBI KS(+) 2x SK by ligation with pBI KS(+). Part of MCS was excised by digest with Kpn I and BamH I (see also a), and the fragment SK-PH I was inserted in return. Using SK-PH I the previously present Bam H I restriction site was masked by means of modification of a base pair (bold letters) and a new BamH I restriction site was introduced at the same time. Due to the different restriction sites (Kpn I/BamH I) the fragment can only be cloned in a~~

possible orientation. The restriction site Pvu I served as a control restriction site for the successful incorporation of the insert SK-PH I (no further data shown in this connection).

c) Diagram of pBI KS(+) 2x SK. PB1 KS(+) 2x SK was formed by ligation of SK-PH I with the BamH I/Kpn I digested pBI KS(+) (cf. b). The modified BamH I restriction site marked by an asterisk could no longer be excised by BamH I. In order to simplify the following text, the region marked in the illustration (SK primer + non-hybridizing sequence) is marked by a black arrow. This leads to the schematic plan for pBI KS(+) 2x SK as shown under item d).

d) Simplified presentation of pBI KS(+) 2x SK. The full black line stands for MCS, the broken line stands for the remaining vector pBI KS(+). The black arrows show the 5' → 3' direction of the cloned SK primers plus 4 bp sequence not to be hybridized (*cf.* item c).

Figure 2: simplified diagram of pBI KS(+) 7x SK.

The pBI vector is marked by a broken black line; seven SK primer sequences are now contained in its Kpn I/Sac I-oriented MCS. The SK-PH II fragment (dashed arrow on the top and sequence "SK-PH II" emphasized by lines at the bottom) inserted the seventh SK primer and the additional Eag I restriction site in the vector. The important sequences are emphasized in detail. The SK primer sequence is light gray, the rest of MCS and the 4 base spacers are dark gray. The restriction sites are marked in the sequence by a finely broken black line.

Figure 3: Diagram of cloning a pBI 2x block plasmid

a) Insertion of a 7x SK block in the Not I-opened pBI 1x block DNA. The characterization of the individual components is identical with those of figure 1 or figure 2. The clone pBI 1x block was linearized with the Not I enzyme and ligated with the PCR fragment subsequently cut by Eag I beforehand. In order to simplify the following text, the seven SK fragments are combined into a gray arrow.

~~b) Presentation of the transitions between individual blocks. The marking of the components can be compared with that in figure 1.a-d. By ligation of the 7x SK block (gray arrow) in the proper orientation, the Not I restriction site which opened the pBI 1x block beforehand was masked by the 5' end of the newly added 7x SK block (bold letters) and could no longer be excised by Not I. The 3' end of the fragment completes the Not I restriction site towards the vector. As a result, it is possible in the next cloning run to again linearize the pBI 2x block with Not I without losing the 14 SK primer. In contrast to the BamH I cleavage site between the individual SK primers in the block (BamH I*), the BamH I restriction site at the 5' end of a 7x block is maintained (BamH I) and can subsequently be used as an orientation control.~~

~~Figure 4: Sequencing result of the plasmid construct containing 27 SK primer elements~~
~~Black bars mark the SK primer sequence regions in the repetitive region sequenced from both sides. Sequences ATCT or GCCG which have a length of 4 base pairs are located between these SK primer sequence regions for reasons of cloning technique.~~

~~Figure 5: Diagram of the labeling experiment~~

The following example explains the invention in more detail.

Example

Methods of producing the plasmids containing the repetitions are well known in the art (see, for example, Sambrook *et al.*, Molecular cloning; a laboratory manual; second edition; Cold Spring Harbor Laboratory Press, 1989; and Current Protocols in Molecular Biology, John Wiley and Sons, 1994-1998). These techniques, including, for example, DNA replication, restriction endonuclease digestion, ligation, agarose gel electrophoresis, and PCR, are known to skilled artisans.

The SK primer element of Bluescript was selected for the repetition (Stragene company, Heidelberg, Germany) because it does not contain any self-complementary or homooligomeric regions, with a G/C content of 50 %**, which [[it]]** lies within the average region of natural DNA and is suited for the almost full production of directly repetitive regions as regards the cloning technique. Furthermore, it is advantageous that this region hybridizes reliably and stably with a complementary sequencing primer (identical with the SK primer ~~described herein~~) since it has been designed by Stratagene (Heidelberg) as a sequencing primer binding site.

A short oligonucleotide fragment is required for the construction of SK primer sequence elements in repetitive succession. It contains the SK primer sequence and restriction sites for carrying out cloning. For this purpose oligonucleotides complementary to one another were synthesized. These ss-DNA fragments were converted by hybridization into ~~clonable~~ **cloneable** ds fragments by juxtaposing the two complementary oligonucleotides in equimolar fashion in 10 mM Tris buffer. A successfully obtained clone was used as a control. The resulting fragments were referred to as SK-PH I (SK primer hybrid I; fragment which was used for the SK primer replication of 2 - 6 SK primer sequences; see ~~Ill. 1~~ **Fig. 1**) and SK-PH II (SK primer hybrid II; fragment which introduced the seventh SK primer and the Eag I restriction site; see ~~Ill. 2~~ **Fig. 2**).

For the production of the plasmid pBI KS(+) (pBluescript KS(+)) with two SK primers (pBI KS(+) 2x SK), the pBI KS(+) had to be **digested** ~~opened~~ using BamH I and Kpn I, part of the multiple cloning site MCS having been removed (~~Ill. 1a~~ **Fig. 1A**). The complete double digest was identified on a 2 % agarose gel, followed by ethanol precipitation. The insert SK-PH 1 (~~Ill. 1b~~ **Fig. 1B**) was added to the **digested** ~~opened~~ vector for ligation in a tenfold excess (see ~~Ill. 1b~~ **Fig. 1B**). This high excess could be justified since the 5' ends of the fragment were not phosphorylated, i.e. oligomers of the inserts could not form. The transformation in *E. coli*, e.g. XL1-Blue, was carried out using this ligation batch. Of the raised colonies the plasmid DNA was isolated by mini-

preparation from three clones for cloning control. The resulting clones are indicated below as pBI KS(+) 2x SK (~~III. 1c~~ **Fig. 1C**).

The further cloning of plasmids with up to seven SK elements contained in equal orientation was time-consuming, since one clone from the last cloning run served in each case as a basis for the next cloning step. Correspondingly, the mini-prep-DNA of the select pBI 2x SK clone was again double-digested by BamH I/Kpn I and admixed with SK-PH I, ligated and transformed in *E. coli* XL1-Blue. Contrary to the strategy used for cloning pBI 2x SK, attention had then to be paid especially to an efficient double digest using BamH I and Kpn I. As shown in ~~III. 1c~~ **Fig. 1C**, the restriction sites into which another SK-PH I fragment should be integrated, were only six base pairs apart from one another. Such a small distance between two restriction sites does not permit the simultaneous restriction of both restriction sites. Correspondingly, the restriction had to be carried out successively using the two enzymes. Cloning up to the plasmid pBI KS(+) 6x SK was carried out in this way.

~~Having cloned~~ **To clone** the seventh SK primer sequence, the repetitive elements were replicated block-wise. This could only function with a restriction site separating the region with seven SK primers as a unit from the vector. This was enabled by ligation of the SK-PH II (~~III. 2~~ **Fig. 2**) in pBI 6x SK. Along with the seventh SK element, SK-PH II introduced the new restriction site Eag I into the vector. The seven SK primers were then confined by two Eag I restriction sites (~~III. 2~~ **Fig. 2**) because the starting vector pBI KS(+) already had such a restriction site in the MCS.

In order to accelerate the further cloning steps, the block-wise replication of the SK elements was made by means of polymerase chain reaction (PCR). The plasmid preparation from XL1-Blue was taken as a template DNA for the amplification of the fragment with seven repetitive elements. It was derived directly from the original colony (pBI 7x SK). **At first, to optimize** ~~In a first optimization of~~ the PCR**, primer pair used to amplify** ~~it should be analyzed which primer pair amplified~~ the target fragment **should be analyzed for** ~~having the best~~ quality and quantity. The primers M13, M13

reverse, T3 and T7 (M13: TGTAAAACGACGGCCAGT (SEQ ID NO:6); M13 reverse: CAGGAAACAGCTATGACC (SEQ ID NO:7); T3: AATTAACCCTCACTAAAGGG (SEQ ID NO:8); T7: TAATACGACTCACTATAGGG (SEQ ID NO:9)) were tested in various combinations. All of these primers had their binding sites outside the MCS, either close to the ß-galactosidase starting point or close to the T7 transcription starting point in pBluescript KS (+). PCR ~~took place~~ **were performed** under standard conditions. ~~The various batches contained the~~ **Various batches containing** matching primers in the various combinations **included,** ~~possible:~~ M13/M13 reverse, T7/T3, T7/M13 reverse and T3/M13. All of the four primers were combined without the template in the negative control. Since the PCR batch supplied the best results with T3/T7, this primer pair was used for the PCR.

In order to be able to ligate the insert fragment obtained by means of PCR into the NotI-**digested** ~~opened~~ vector, ~~it had to have~~ **Not I compatible** sticky ends **are needed** ~~compatible with Not I~~. For this purpose, the PCR fragment which contained the seven SK primers had to be subsequently cut at the edges. The restriction enzyme Eag I shortened the 246 bp long PCR fragment whose edges **were** closed **to** the sequences of primers T3/T7 by **47 bp / 51 bp, respectively** ~~47 bp and by 51 bp on the other side~~. This difference with respect to the control could still be made visible using a 2.2 % gel. pBI KS(+) 7x SK changed into pBI KS(+) 14x SK in only one step using the PCR-amplified 7x SK fragment whose edges became compatible with Not I by Eag I digest. See **Figures 3A and 3B** ~~Ill. 3~~ for an outline of the cloning **strategy** ~~manner~~. The digested fragment was purified prior to ligation by the PCR purification kit (Qiagen company). This should serve for removing the **unused** primers ~~not consumed~~ in the PCR reaction and the fragments resulting from the **digestion** ~~digest~~.

As compared to the first cloning steps, which resulted in pBI KS(+) 7x SK, the vector was not **digested** ~~opened~~ by two different enzymes (Kpn I/BamH I; see **Fig. 1** ~~Ill. 1~~) but linearized by Not I. Therefore, an accumulation of religations had to be expected. In this cloning, a religation could not be counteracted by an insert concentration increased many times over (7x SK fragment), since the DNA blocks were phosphorylated at their

5' ends and uncontrollable oligomerizations of the insert DNA had to be expected. Therefore, the religations were reduced, or even suppressed, by dephosphorylating the vector. The DNA pBI KS(+) 7x SK, as described above, is designated as the pBI 1x block below.

The pBI 1x block **digested ~~opened~~** by Not I was ligated with the purified PCR fragment which also contained seven SK primers. This was enabled by the single Not I restriction site located at the edge of the repetitive elements, by which the pBI 1x block was linearized. As mentioned above, the PCR fragment was subsequently cut with the Eag I enzyme compatible with Not I and ligated directly to the seven SK primers of the vector (pBI 2x block).

Since pBI 7x SK proved to be stable in the JM110 host strain, the ligation batch of the plasmid with 14 SK elements was also transformed into this strain. The transformation of pBI KS(+) 14x SK in JM110 yielded 118 transformants. This corresponded to a transformation rate of 1.7×10^3 cfu (colony forming units)/ µg DNA.

Step-wise replication was used, in this case with the aim of building up with the 7x SK blocks a plasmid having 28 repetitive SK primer sequences. For this, the pBI 2x block was linearized by Not I as shown analogously in **Figures 3A & 3B** **~~Ill. 3~~**. The full digest was examined on a 1 % agarose gel. The 5' ends of this Not I- **digested ~~opened~~** plasmid were dephosphorylated and ligated with the 7x SK block. Seven colonies resulted from this transformation.

Control digest with BamH I of several candidate clones showed that a complete 7x SK block had additionally been inserted. One of the clones was replicated for a mini preparation and the DNA was prepared. The sequence analysis from this mini preparation identified the complete and correct sequence of 21 SK primers including the functioning restriction sites which were required for **subsequent ~~the next~~** cloning run. The gel analyses were confirmed in this connection. This clone is referred to as pBI 3x block below. It served as a precursor for **subsequent ~~the next~~** insertion run.

In order to obtain a plasmid having 28 repetitive SK elements, the block-wise replication of the 7x SK block was continued. The pBI 3x block was used as a starting plasmid of this cloning. This cloning was treated like the two preceding ones. The pBI 3x block was linearized using Not I, checked for full digest in a 1 % agarose gel and then dephosphorylated. The dephosphorylated vector was used together with the PCR-amplified and subsequently cut 7x SK block in a ligation batch. The control ligation for evaluating the dephosphorylation yielded 2 clones. **A total of** 59 clones **were** formed in the transformation of the ligation with insert, 16 colonies of which were selected for a mini preparation. Separation with an agarose gel after Sac I/Kpn I digest was made as usual on a 2.2 % gel matrix.

A total of 5 clones had prolonged insert regions. Control digests with BamH I and triple digests with Sac I/Kpn I/BamH I showed fragment patterns indicating that no complete 7x SK block might have joined. A BamH I site must have been deleted in the newly joined block during the cloning instead.

One of the five equal clones was chosen and a sequence analysis was made using its mini-prepared DNA. Sequencing confirmed the result that the newly joined BamH I restriction site was deleted. The complete SK primer with intact BamHI restriction site of the last joined 7x SK block lacked. The result was a pBI KS(+) plasmid having 27x SK primers. The sequence of this clone is shown in figure 4.